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Estrogen regulates the expression of *N*-methyl-D-aspartate (NMDA) receptor subunit epsilon 4 (Grin2d), that is essential for the normal sexual behavior in female mice

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ABSTRACT

Estrogen plays important roles in the reproductive behavior of animals. In the present study, we found that the Grin2d gene of mouse possessed half-sites of the estrogen-responsive element (ERE) in the 3′-untranslated region (UTR). Quantitative PCR analysis showed that the reduced Grin2d mRNA expression in the hypothalamus of the ovariectomized mice was restored by estrogen administration. Downregulation of Grin2d mRNA expression was also detected in the hypothalamus of estrogen receptor alpha-knockout female mice. Moreover, estrogen-induced lordosis response was decreased in Grin2d-knockout mice. These results suggest that estrogen regulates lordosis behavior through the regulation of Grin2d expression in the hypothalamus of female mice.

1. Introduction

Estrogen plays important roles in the development of the brain and its functioning. Experimental models using female mice and rats showed that estrogen elicits lordosis behavior, that is, the posture exhibited by sexually receptive female rodents. The ventromedial nucleus of the hypothalamus (VMH) and the medial preoptic area are considered to be the main regions of the brain that control the lordosis behavior. These regions of the brain express estrogen receptors (ERs, namely, ERα and ERβ), which are the members of the nuclear receptor family of ligand-activated transcription factors [1]. The ERα subtype is predominant in the VMH, and it appears that ERα plays a major role in the exhibition of female receptivity defined as the lordosis response. Studies on ERα-knockout mice indicated the critical role of ERα in female sexual behavior [2–4]; estrogen-treated ovariectomized ERα-knockout mice did not display lordosis response [3]. Moreover, a previous study that used subtype-selective agonists for ERα and ERβ revealed that ERα is primarily involved in the elicitation of receptive and proceptive behaviors in female mice [5].

It is well known that estrogen affects brain functions; however, the target genes under the direct transcriptional control of ERα remain to be clarified. While investigating estrogen receptor-binding sites in the human CpG island library, we isolated a genomic DNA fragment named EB11, which corresponds to the 3′-untranslated region (UTR) of the gene coding for the human *N*-methyl-D-aspartate (NMDA) receptor subunit 2D, i.e., GRIN2D [6,7]. EB11 contains nine half-sites of the estrogen-responsive element (hERE) within 600 bp, which are recognized by ERα. A relatively large number of hEREs are also conserved in the 3′-UTR of Grin2d in rats [7]. It has been demonstrated that in rats, the hERE-containing region activates ERα-mediated transcription, and estrogen upregulates the mRNA expression of Grin2d in the hypothalamus [7]. Grin2d, which was originally isolated from mice and named as epsilon 4,

Abbreviations: UTR, untranslated region; ERE, estrogen-responsive element; hERE, half-site of estrogen-responsive element; NMDA, *N*-methyl-D-aspartate; Grin2d, NMDA receptor subunit 2D/epsilon 4; PCR, polymerase chain reaction

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consists of an NMDA receptor channel along with NMDA receptor type 1 (Grin1) [8,9]. Interestingly, Grin2d mRNA was co-expressed with ERα in the mouse and rat hypothalamus, thereby suggesting that there exists some functional relationship between Grin2d and ERα [7,10]. In the present study, we found that the hERE motifs in the 3′-UTR of Grin2d are conserved in mice. The Grin2d mRNA expression in mice was regulated by estrogen in the hypothalamus, and Grin2d-knockout mice exhibited attenuated sexual behavior, implying that Grin2d plays a key role in brain functioning by mediating estrogen activity.

2. Materials and methods

2.1. Animals and estrogen treatment

All animal experiments were approved by the Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from CLEA Japan. ERα-knockout mice were obtained from Jackson laboratory and maintained on a C57BL/6 background. Two groups of 4-month-old female mice were ovariectomized and injected subcutaneously (s.c.) with either 17β-estradiol dissolved in sesame oil (10 μg/kg) (OVX + E group) or sesame oil (OVX + Veh group). The sham-operated control group (Sham + Veh) was administered only sesame oil. After 8 h, the mice were sacrificed by decapitation, and the hypothalamus and preoptic area were immediately isolated and stored at −80 °C. Similar procedure was performed to isolate the brain regions from 4-month-old female ERα-knockout mice that were not administered 17β-estradiol treatment. It has been reported that the concentration of plasma 17β-estradiol is much lower in OVX mice compared with that in intact females [11]. In ERα-knockout female mice, estrogen insensitivity has been observed by failure of 17β-estradiol treatment to induce target gene expressions [12]. In addition, they have 10-fold higher concentration of plasma 17β-estradiol compared with that observed in wild type females [13].

2.2. RNA purification and quantitative polymerase chain reaction (PCR)

Total RNA was extracted from the hypothalamus by using the Isogen reagent (Nippon Gene). Quantitative real-time polymerase chain reaction (qPCR) was performed according to a method described in a previous report [14]. The relative amount of PCR product was calculated using the comparative cycle threshold (CT) method, and GAPDH was used as the external control. The experiments were performed in triplicate. The mean values and standard deviation were calculated, and the statistical significance was determined using one-way ANOVA and Scheffe's post-hoc tests ($P < 0.01$).

2.3. Analysis of sexual behavior

Six-month-old female Grin2d-knockout mice [15] ($n = 12$) and wild type mice ($n = 12$) were bilaterally ovariectomized (OVX) under anesthesia 1 week before hormonal treatment. The female mice were primed twice with 17β-estradiol (20 μg/body, s.c.) at 48 and 72 h before testing, and once with progesterone (100 μg/body, s.c.) at 4 h before testing. Female mice were paired with sexually experienced males for behavioral testing. Each test was continued until a female received 10 mounts from a stud male. All behavioral tests were performed during the dark phase under red light, and the results were analyzed by a blinded observer who was kept unaware of the treatment group of the mice. The behavior of each female mouse toward the 10 male approaches or attempted mounts was rated as follows: (i) rejective (i.e., flee, kick, upright posture), (ii) pre-receptive posture (i.e., receptive still posture without lordosis), or (iii) lordosis response [16]. The percentage of a particular response [(response/total mounts allowed) × 100] was calculated for each female mouse, and the statistical significance was determined by using Mann–Whitney U non-parametrical test ($P < 0.01$).

3. Results

3.1. Conservation of estrogen-responsive element (ERE) motifs in the 3′-UTR of Grin2d in mice

In humans, the genomic fragment EB11, which is a part of 3′-UTR of the Grin2d gene, contains nine direct repeats of hERE and strongly binds to the recombinant ERα protein in vitro [6,7]. In this study, we compared the sequences of EB11 and the corresponding region in Grin2d gene in mice. As shown in Fig. 1, the gene sequence in mice exhibited significant homology with EB11; the former contained eight hEREs, among which, three were conserved with respect to their position when compared to EB11. These findings indicate that the hERE-containing region of Grin2d in mice may play an important role in the estrogen responsiveness of the gene.

3.2. Estrogen-inducible expression of Grin2d mRNA in the hypothalamus of female mice

Next, we used ovariectomized mice to examine whether Grin2d expression is regulated by estrogen in the hypothalamus. At 2 weeks after surgery, 10 μg/kg 17β-estradiol was s.c. administered; subsequently, the total RNA was extracted from the hypothalamus after 8 h. The total RNA was also extracted from the hypothalamus obtained from ERα-knockout mice. As shown in Fig. 2, the expression levels of Grin2d mRNA were low in the OVX + Veh groups as compared to those in the Sham + Veh groups. However, the expression level of Grin2d mRNA in the hypothalamus was increased in the OVX + E groups as compared to that in the OVX + Veh groups. In addition, the expression level of Grin2d mRNA in the hypothalamus was low in ERα-knockout mice and in the mice of the OVX + Veh groups. The regulation of the mRNA expression of the gene encoding the progesterone receptor (PR), which is known to be an estrogen-responsive gene, appeared to be similar to the regulation of Grin2d by 17β-estradiol. As reported previously, OVX mice have lower plasma concentration of 17β-estradiol compared with that in intact female mice at all stages of estrus cycle [11]. In addition, ERα-knockout female mice have dysfunctional ovaries [13] and show estrogen insensitivity though the serum levels of 17β-estradiol are more than 10-fold higher than those in the wild type females [12]. Hence, these results indicate that Grin2d expression is upregulated by estrogen via ERα in the hypothalamus of female mice.

3.3. Differential regulation of the NR subunit expression by estrogen in the hypothalamus

Next, we investigated whether other members of the NMDA receptor family, i.e., Grin1, Grin2a, Grin2b, and Grin2c are regulated by 17β-estradiol in the hypothalamus (Fig. 3). The results showed that the mRNA expression of Grin1, Grin2a, Grin2b, and Grin2c was not regulated by 17β-estradiol, while that of Grin2a was downregulated in the hypothalamus in the OVX + Veh groups. The expression of Grin2a mRNA did not change significantly among the Sham + Veh, OVX + E, and ERα-knockout groups, thus suggesting that Grin2a may be modulated by a factor other than ERα. In conclusion, the expression of the gene encoding the NMDA receptor subunit is regulated by estrogen in a complex manner.

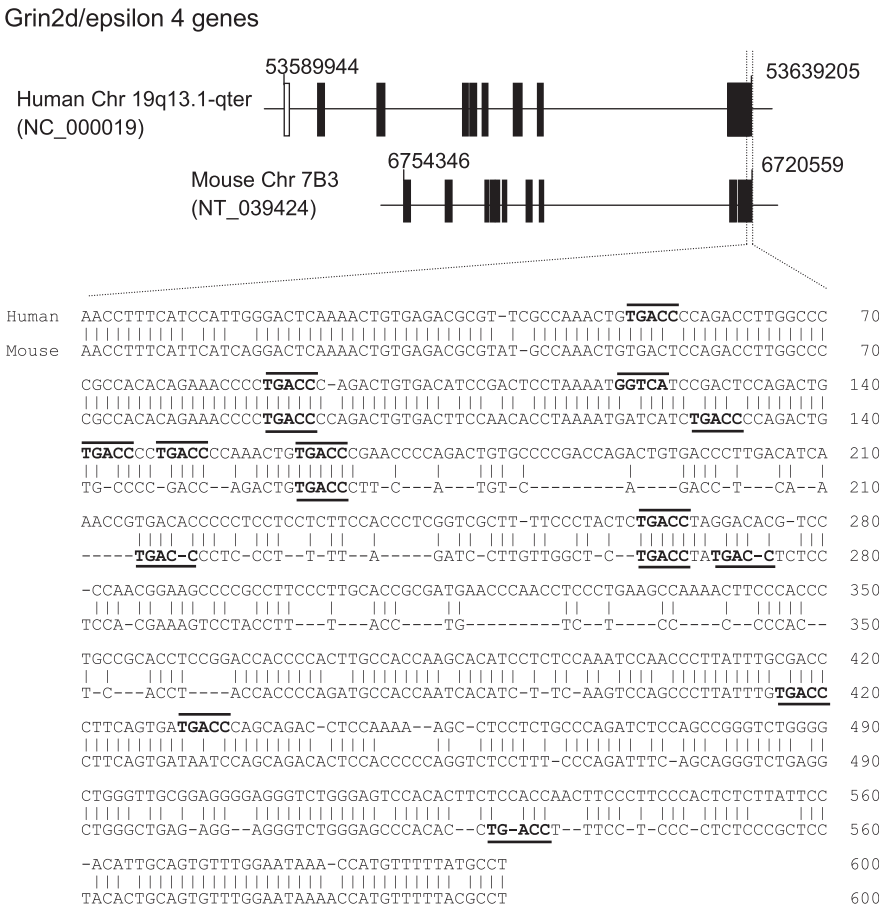

Fig. 1. Conservation of EREs in the Grin2d gene in mice. The genomic sequences surrounding the mouse Grin2d gene (GenBank accession no. NT_039424) and human GRIN2D gene (GenBank accession no. NC_000019) were retrieved from the GenBank database. Their genomic structures are represented schematically; the coding and non-coding exons are indicated by solid and open boxes, respectively. The comparison between the 3′-UTRs of Grin2d in mice and GRIN2D in humans is shown at the bottom of the figure. The hERE sequences are indicated in bold, underlined in the mouse, and over-lined in the human. The 3′-UTR of Grin2d in mice contains eight hEREs, three of which are located at conserved positions as compared to the corresponding positions in human.

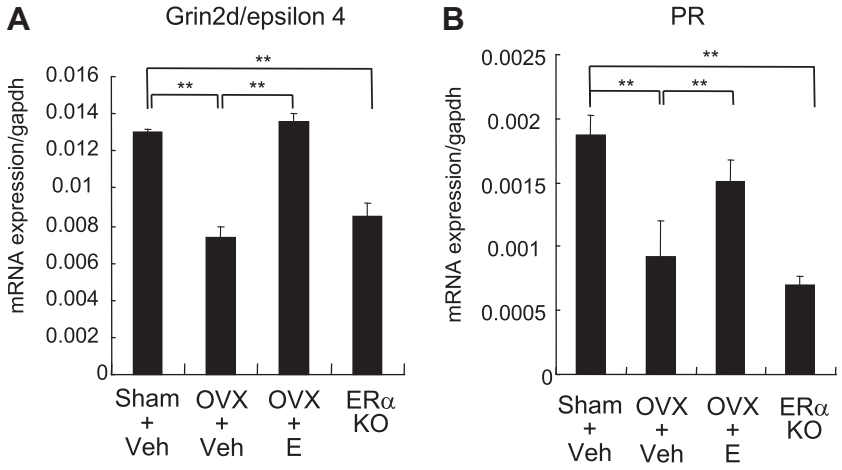

Fig. 2. Estrogen regulation of Grin2d expression in the hypothalamus of female mice. Ovariectomized mice were subcutaneously administered 10 μg/kg of 17β-estradiol (OVX + E group) or the vehicle (OVX + Veh group). Sham-operated mice were injected subcutaneously with the vehicle (Sham + Veh). At 8 h after 17β-estradiol or vehicle administration, the hypothalamus was isolated for total RNA purification. The total RNA isolation was also isolated from the hypothalamus of ERα-knockout mice. The mRNA levels of Grin2d (A) and progesterone receptor (B) were measured by quantitative polymerase chain reaction (qPCR). The results are shown as mean ± standard deviation (S.D.). $^{**}P < 0.01$ (by one-way ANOVA and Scheffe's post-hoc tests).

3.4. Attenuation of lordosis response in Grin2d-knockout mice

To further elucidate the function of Grin2d in vivo, we examined the lordosis response of Grin2d-knockout mice [15]. As shown in Fig. 4, the display of lordosis in response to the mounting of a stud male was clearly impaired in Grin2d-knockout female mice that were treated with 17β-estradiol and progesterone. Moreover, the frequency of rejective posture was high in the Grin2d-knockout

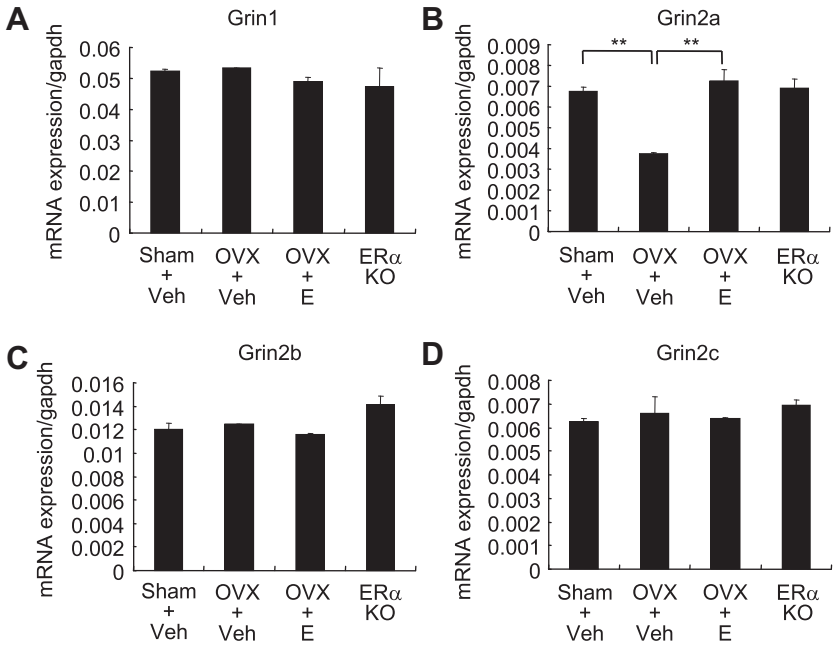

Fig. 3. Regulation of Grin1, Grin2a, Grin2b, and Grin2c expressions by estrogen in the hypothalamus of female mice. The mRNA levels of Grin1, Grin2a, Grin2b, and Grin2c were evaluated by qPCR using the cDNAs as described in Fig. 2. The results are shown as mean ± S.D. $^{**}P < 0.01$ (by one-way ANOVA and Scheffe's post-hoc tests).

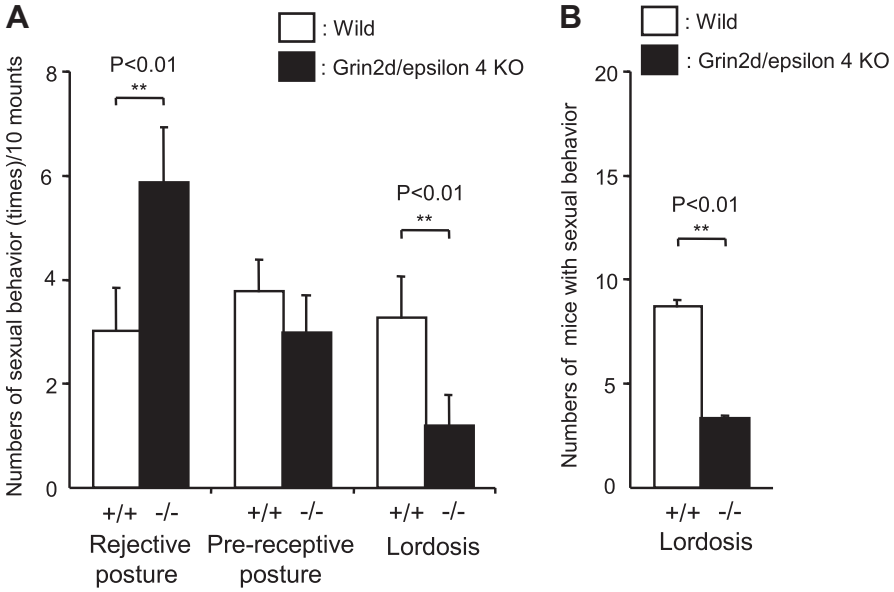

Fig. 4. Reduced sexual behavior in Grin2d-knockout female mice. Grin2d-knockout ($n = 12$) and wild type ($n = 12$) female mice were administered 10 μg/body 17β-estradiol twice (i.e., first at 48 h and then at 72 h before lordosis testing) and 100 μg/body progesterone once before lordosis testing. The frequency and intensity of lordosis postures in response to the first 10 mounts by the male mouse were recorded as follows: rejective, totally unreceptive; pre-receptive, proceptive/still posture; and lordosis, receptive lordosis posture. The results are expressed as the mean ± S.D. $^{**}P < 0.01$ (by Mann–Whitney *U* non-parametrical test).

female mice. The number of Grin2d-knockout mice showing sexual behavior was significantly lower than that of the wild type animals. These results indicate that Grin2d is involved in the lordosis response elicited in estrogen-primed mice.

4. Discussion

In the present study, we found that the 3′-UTR of Grin2d in mice contains a high number of hERE motifs, similar to those in the GRIN2D gene in humans. The expression level of Grin2d mRNA in the hypothalamus of ovariectomized female mice was high after 17β-estradiol treatment, whereas its expressions in the hypothalamus of ERα-knockout mice and in ovariectomized female mice injected with the vehicle alone were low. These results suggest that estrogen regulates the mRNA expression of Grin2d in the hypothalamus, at least in part, in an ERα-dependent manner. Since it has been demonstrated that the conserved sequence of the 3′-UTR of GRIN2D gene in human exhibits enhancer activity in the presence of ERα, the corresponding sequence in mice may influence estrogen-regulated transcription of Grin2d. In addition, the ERE-containing region could function as a potential site for crosstalk between estrogen and thyroid hormone signaling pathways since

the corresponding region in rats is regulated by both the ERs and thyroid hormone receptors [17].

A sexual behavior test demonstrated that Grin2d-knockout female mice display low lordosis response after 17β-estradiol administration. Although Grin2d-knockout mice show reduced spontaneous activity in an open-field test [15], increased rejective posture was observed in our sexual behavior test. Hence, we assume that Grin2d plays a specific role in eliciting lordosis response in female mice. Because 17β-estradiol induces Grin2d expression in the hypothalamus of female mice, as discussed above, it has been suggested that Grin2d mediates estrogen-induced lordosis and acts as a direct estrogen target in the hypothalamus, which is known to be the key site that controls female sexual receptivity [18]. It is believed that the activation of NMDA receptors in the hypothalamus is critical for stimulating the secretion and expression of the gonadotropin-releasing hormone (GnRH) [19,20] that is necessary for the regulation of reproductive function. We speculate that Grin2d may play a role in GnRH regulation. Studies on immortalized hypothalamic GT1-7 neurons expressing Grin2d revealed that GnRH is released by the NMDA receptor since it is completely blocked by a specific NMDA receptor antagonist [21]. In the adult hypothalamus, estrogen induces a transient increase in the density of dendritic spines via an NMDA receptor-dependent mechanism [22,23]. Estrogen also increases the sensitivity of pyramidal cells to the NMDA receptor-mediated synaptic input [24] and promotes axon growth of VMH-derived neurons. These findings implicate that estrogen regulates the amount/activity of NMDA receptor for controlling reproductive activity through synaptic patterning.

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